International Standard

ISO 6579-4

First edition
2025-01

Microbiology of the food chain — Horizontal method for the detection, enumeration and serotyping of *Salmonella* —

Part 4: Identification of monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-) by polymerase chain reaction (PCR)

Microbiologie de la chaîne alimentaire — Méthode horizontale pour la recherche, le dénombrement et le sérotypage des Salmonella —

Partie 4: Identification du variant monophasique de Salmonella *Typhimurium (1,4,[5],12:i:-) par réaction de polymérisation en chaîne (PCR)*

Reference number
ISO 6579-4:2025(en)

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Published in Switzerland

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Foreword

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This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*, in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 463, *Microbiology of the food chain*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

A list of all parts in the ISO 6579 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

In several international, regional and national laws, regulatory limits are set to ensure the so-called "absence" of *Salmonella* spp. in samples of the food chain. Moreover, several European Commission (EC) regulations also demand the absence of particular *Salmonella* serovars which have shown to cause a relatively high percentage of human salmonellosis. One of these *Salmonella* serovars for which legal criteria are set is *Salmonella* Typhimurium, including its monophasic variant 1,4,[5],12:i:- (e.g. Regulation (EC) No. 1086/2011[10]). Hence, it is important to know that a serovar found with antigenic formula 1,4,[5],12:i:- is indeed the monophasic variant of *Salmonella* Typhimurium (1,4,[5],12:i:1,2) and not the monophasic variant of another *Salmonella* (*S.*) serovar for which no criteria are set, such as *S*. Lagos (1,4,[5],12:i:1,5), *S*. Agama (4,12:i:1,6), *S.* Farsta (4,[5],12:i:e,n,x), *S*. Tsevie (1,4,12:i:e,n,z_{15}), *S.* Gloucester (1,4,12,27:i:l,w) or *S*. Tumodi (1,4,12:i:z_6). Confirmational distinction between *Salmonella* Typhimurium and *Salmonella* non-Typhimurium serovars can be determined using molecular analysis, such as the PCR technique(s) described in this document.

Microbiology of the food chain — Horizontal method for the detection, enumeration and serotyping of *Salmonella* —

Part 4: Identification of monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-) by polymerase chain reaction (PCR)

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for detecting, enumerating and (sero)typing *Salmonella* are only undertaken in properly equipped laboratories, under the control of a skilled microbiologist, and that great care is taken in the disposal of all incubated materials. Persons using this document should be familiar with normal laboratory practice. This document does not purport to address all of the safety aspects, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices.

1 Scope

This document specifies a horizontal *in vitro* method for the molecular identification and differentiation of the monophasic variant of *Salmonella enterica* subsp. *enterica* serovar Typhimurium (1,4,[5],12:i:-) lacking the second H phase H:1,2, starting from isolates. The method detects specific DNA sequences of an intergenic region of the first H phase flagellin cluster for identification of *Salmonella enterica* subsp. *enterica* serovar Typhimurium (further called *Salmonella* Typhimurium) and specific DNA sequences of genes associated with second H phase flagellar antigen expression.

The method is applicable for:

— differentiation of the isolate under analysis between monophasic *Salmonella* Typhimurium and the monophasic variant of another *Salmonella* non-Typhimurium serovar that has the same antigenic formula;

— identification of the isolate under analysis being either monophasic *Salmonella* Typhimurium or (biphasic) *Salmonella* Typhimurium.

This document is applicable for the analysis of a pure culture belonging to the genus *Salmonella*, isolated from:

— products intended for human consumption;

— products intended for animal feeding;

— environmental samples in the area of food and feed production and handling;

— samples from the primary production stage.

This document can also be applied in other domains for identification of monophasic *Salmonella* Typhimurium (e.g. environmental, human health, animal health).

NOTE This method has been validated in a method evaluation study and in an interlaboratory study with a large set of different strains (target and non-target strains), isolated from different sources (food products, animals, animal feed, primary production samples and humans). For detailed information on the validation, see Annex E.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 7218, *Microbiology of the food chain — General requirements and guidance for microbiological examinations*

ISO 11133, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media*

ISO 20836, *Microbiology of the food chain — Polymerase chain reaction (PCR) for the detection of microorganisms — Thermal performance testing of thermal cyclers*

ISO 22174, *Microbiology of the food chain — Polymerase chain reaction (PCR) for the detection and quantification of microorganisms — General requirements and definitions*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 22174 and the following apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
monophasic *Salmonella* Typhimurium
variant of *Salmonella enterica* subsp. *enterica* serovar Typhimurium lacking the second H phase H:1,2, having the antigenic formula 1,4,[5],12:i:-

3.2
presumptive monophasic *Salmonella* Typhimurium
pure culture characterized as belonging to the genus *Salmonella*, giving a positive reaction for O-antigen O:4 and H-antigen H:i and with a negative reaction for the second H phase H:1,2

3.3
threshold cycle crossing point
point of the amplification curve at which the fluorescence signal rises above the baseline or crosses a predefined threshold setting

4 Principle

4.1 General

The identification of the monophasic variant of *Salmonella* Typhimurium comprises the three successive steps described in 4.2 to 4.4, starting with a pure culture characterized as belonging to the genus *Salmonella*.

4.2 Preparation of well-isolated colonies

The culture is streaked onto the surface of a (pre-dried) non-selective agar medium and incubated between 34 °C and 38 °C for 24 h, to obtain well-isolated colonies.

4.3 Suspension of a colony

A well-isolated colony is selected and suspended in 100 µl saline solution (0,85 % m/v) or in 100 µl PCR grade water.

4.4 Amplification and detection

The suspended bacterial cells are analysed by PCR for detection of the genetic sequences unique to *Salmonella* Typhimurium (1,4,[5],12:i:1,2) and its monophasic variant lacking the second H phase (1,4,[5],12:i:-), as well as for detection of specific genetic sequences of genes associated with the second H phase flagellar antigen expression. Specific PCR assays including primers and probes are described in Annexes B to D.

5 Culture media and reagents

Follow current laboratory practices in accordance with ISO 7218. For the steps in 4.3 and 4.4, use reagents and consumables of quality suitable for molecular biological applications (see ISO 22174). The composition of culture media and reagents and their preparation are specified in Annex A. For performance testing of culture media, follow the procedures in accordance with ISO 11133 and Clause A.4. The primers and probes for identification of the monophasic variant of *Salmonella* Typhimurium (1,4,[5],12:i:-) are listed in Annexes B to D.

6 Equipment and consumables

Disposable equipment is an acceptable alternative to reusable glassware if it has suitable specifications. The usual microbiological laboratory equipment (see ISO 7218) and molecular biology equipment (see ISO 22174) and, in particular, the following shall be used.

6.1 Incubator, capable of operating in the range of 34 °C to 38 °C.

NOTE The range 34 °C to 38 °C for incubation of culture media includes the use of incubators set at 35 °C ± 1 °C, 36 °C ± 2 °C or 37 °C ± 1 °C.

6.2 Sterile loops, of approximate diameter 3 mm (10 µl) or 0,3 mm (1 µl), or an inoculation needle/wire.

6.3 Water bath, capable of operating at 47 °C to 50 °C.

6.4 Refrigerator, capable of operating at 5 °C ± 3 °C.

6.5 Drying cabinet or **oven**, capable of operating between 25 °C and 50 °C.

6.6 pH-meter, having an accuracy of calibration of ± 0,1 pH unit at 20 °C to 25 °C.

6.7 Equipment for suspension of a colony, e.g. (micro)centrifuge tubes.

6.8 Graduated pipettes and **pipette filter tips**, for handling volumes between 0,2 µl and 13,55 µl, depending on the PCR assay used (see Annex B, C or D). For more reactions per mix, larger volumes are needed.

6.9 Vortex mixer or equivalent.

6.10 Sterile Petri dishes, with a diameter of approximately 90 mm.

6.11 Equipment for PCR and **real-time PCR**, e.g. microcentrifuge or plate spinner.

6.12 Thermal cycler or **real-time PCR thermal cycler**, calibrated in accordance with ISO 20836.

6.13 Associated consumables for conventional or real-time PCR, e.g. PCR tubes, optical plates and seals, optical plate holder, suitable for use with the selected PCR machine (see Annex B, C or D).

6.14 Apparatus for dry sterilization (oven) or **wet sterilization (autoclave)**, as specified in ISO 7218.

7 Presumptive monophasic *Salmonella* Typhimurium

The isolate to be used for further identification shall be a pure culture characterized as belonging to the genus *Salmonella* (see ISO 6579-1). A presumptive monophasic *Salmonella* Typhimurium will show a positive reaction for O-antigen O:4 and H-antigen H:i and a negative reaction for the second H phase (see ISO/TR 6579-3).

8 Culturing the isolate

Streak the culture of Clause 7 (e.g. with a 10 µl loop; 6.2) on the surface of a non-selective agar medium (e.g. nutrient agar; Clause A.2) to obtain well-isolated colonies. Incubate the plates, inverted, between 34 °C and 38 °C (6.1) for 24 h ± 3 h.

9 Procedure

9.1 Preparation of cell suspension or DNA

By means of an inoculating wire or a sterile loop (6.2), pick and suspend (a portion of) one colony in 100 µl saline solution (0,85 % m/v; Clause A.3) or in 100 µl PCR grade water in an appropriate tube (6.7).

Mix (6.9) for homogenization of the suspension.

An aliquot of 2 µl or 2,5 µl, depending on the specific PCR assay, of this bacterial cell suspension is used (see Table B.2, C.2, D.2, D.3 or D.4).

It is also possible to use a DNA extract for the PCR assay. For DNA extraction, for example, thermal cell lysis can be used, or another appropriate extraction method. If shown to be reliable, commercial kits can also be used for DNA extraction, following the manufacturer's instructions.

9.2 PCR amplification and detection

9.2.1 General

Different protocols for multiplex probe-based real-time PCR, multiplex PCR followed by agarose gel electrophoresis detection of the amplification products or single target PCR followed by agarose gel electrophoresis detection can be used.

A probe-based multiplex real-time PCR assay is given in Annex B.

An agarose gel-based multiplex PCR assay is given in Annex C.

An agarose gel-based single target PCR assay is given in Annex D.

Follow all requirements, including the use of suitable equipment (6.11), for the (real-time) PCR amplification as specified in ISO 22174.

9.2.2 PCR controls

Use (process) controls for the PCR assays in accordance with ISO 22174.

For the real-time PCR (see Annex B) and the single target PCR (see Annex D), an internal amplification control (IAC) shall also be used as the targets could all be negative. Since the multiplex PCR (see Annex C) will always result in a PCR fragment (1 000 bp or 250 bp), this procedure does not require an IAC.

10 Expression of results

The results obtained, including controls specified in ISO 22174, shall be unambiguous otherwise the PCR shall be repeated.

The PCR result will be either:

a) positive, if a specific PCR product has been detected and all the controls give expected results, or

b) negative within the limits of detection, if a specific PCR product has not been detected, and controls give expected results.

If the PCR assay identifies the isolate as monophasic *Salmonella* Typhimurium, report the result preferably by giving the antigenic formula as determined.

It is possible that an isolate is phenotypically identified as monophasic *Salmonella* Typhimurium, but genotypically (with PCR) as biphasic *Salmonella* Typhimurium. This can be caused by the fact that the genes are present, but phenotypically not expressed. For identification of these isolates, the PCR results take precedence over serum agglutination test results.

11 Performance characteristics of the method

11.1 Validation in accordance with ISO 17468

The PCR methods described in Annexes B, C and D were validated in accordance with ISO 17468. All relevant data as obtained in steps 1 to 5 of ISO 17468, as well as the results of the interlaboratory study (step 6 in ISO 17468) were reported in Reference [8].

The performance characteristics of the three PCR methods (inclusivity and exclusivity) were determined in a method(s) evaluation study (described in 11.2.1) and in an interlaboratory study (described in 11.2.2).

11.2 Performance characteristics

11.2.1 Method(s) evaluation study

The three PCR assays described in Annexes B, C and D were tested in a method evaluation study, by analysing 172 different strains (target and non-target strains), isolated from different sources (food products, animals, animal feed, primary production samples and humans), in two different laboratories. For the inclusivity and the exclusivity testing, the typing results of *Salmonella* found by slide agglutination were compared to the typing results found by each PCR method.

All data are given in Annex E and more details can be found in Reference [8].

It depends on the intended specific purpose for which the PCR assay is being applied and its performance evaluated, as to whether only monophasic *Salmonella* Typhimurium is considered as target strain (and thus part of the inclusivity study) or if (biphasic) *Salmonella* Typhimurium is also considered as target strain.

If the intended purpose is to determine if the strain under analysis is the monophasic variant of *Salmonella* Typhimurium and not the monophasic variant of another *Salmonella* non-Typhimurium serovar, then monophasic *Salmonella* Typhimurium as well as (biphasic) *Salmonella* Typhimurium can be considered as target strains and the three PCR assays described in Annexes B, C and D perform equally well for identification of monophasic *Salmonella* Typhimurium strains (see Table E.1).

If the aim is to identify whether the strain under analysis is either monophasic *Salmonella* Typhimurium or (biphasic) *Salmonella* Typhimurium, then *Salmonella* Typhimurium should be considered as non-target strain. For this purpose, the gel-based multiplex PCR (see Annex C) can be less specific for some strains than the other two PCR assays (see Table E.2), as this assay is less suitable to distinguish biphasic from monophasic *Salmonella* Typhimurium.

11.2.2 Interlaboratory study

The performance characteristics of each PCR method (see Annexes B, C and D) were determined in an interlaboratory study (step 6 in ISO 17468) to determine the inclusivity and exclusivity of the three methods,

following the procedures described in ISO 16140-6. Details about the interlaboratory study and a summary of the data are given in Clause E.2 for each PCR assay.

A summary of the inclusivity and exclusivity data is given in Table E.4.

In the inclusivity study, pure target strains to be detected by the method were tested. For this interlaboratory study, monophasic *Salmonella* Typhimurium was considered as the only target strain.

In the exclusivity study, pure non-target strains that are not expected to be detected by the method but can potentially be cross-reactive were tested. For this interlaboratory study, *Salmonella* serovars other than monophasic *Salmonella* Typhimurium, including (biphasic) *Salmonella* Typhimurium and other *Enterobacteriaceae* were considered as non-target strains.

12 Test report

The test report shall specify at least the following:

— the test method used, with reference to this document, i.e. ISO 6579-4;

— all operating conditions not specified in this document, or regarded as optional or informative (including informative annexes), together with details of any incidents which can have influenced the test result(s);

— any deviations from this document;

— all information necessary for the complete identification of the sample;

— the test result(s) obtained;

— the date of the test.

13 Quality assurance

The laboratory should have a quality control system to ensure that the equipment, reagents and techniques are suitable for the method. The use of positive controls, negative controls and blanks are part of the method. Performance testing of culture media is specified in Clause A.4 and described in ISO 11133.

Annex A
(normative)

Culture media and reagents

A.1 General

The general specifications of ISO 11133 are applicable to the preparation and performance testing of the culture media described in this annex. If culture media or reagents are prepared from dehydrated complete media/reagents, or if ready-to-use media/reagents are used, follow the manufacturer's instructions regarding preparation, storage conditions, expiry date and use.

The shelf life of the media indicated in this annex has been shown in some studies. The user shall verify this under their own storage conditions (as specified in ISO 11133).

Performance testing of culture media is described in Clause A.4.

A.2 Nutrient agar (example of non-selective agar medium)

A.2.1 Composition

Meat extract	3,0 g
Peptone[a]	5,0 g
Sodium chloride (NaCl) (optional) (CAS Registry Number®[c] 7647-14-5)	5,0 g
Agar	9,0 g to 18,0 g[b]
Water	1 000 ml

[a] For example, enzymatic digest of casein.

[b] Depending on the gel strength of the agar.

[c] Chemical Abstracts Service (CAS) Registry Number® is a trademark of the American Chemical Society (ACS). This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

A.2.2 Preparation

Dissolve the components or the dehydrated complete medium in the water by heating, with frequent agitation.

Adjust the pH (6.6), if necessary, so that after sterilization, it is 7,0 ± 0,2 at 20 °C to 25 °C.

Transfer the culture medium into tubes or flasks of appropriate capacity.

Sterilize for 15 min in the autoclave (6.14) set at 121 °C.

A.2.3 Preparation of nutrient agar plates

Cool the medium to 47 °C to 50 °C in a water bath (6.3), mix, and pour into sterile Petri dishes up to a volume of approximately 15 ml to 20 ml in dishes with a diameter of approximately 90 mm (6.10). Allow to solidify.

Immediately before use, dry the agar plates carefully (preferably with the lids off and the agar surface downwards) in a drying cabinet or oven set between 25 °C and 50 °C (6.5) until the surface of the agar is dry.

Store the poured plates protected from drying, at 5 °C (6.4) for up to four weeks.

A.3 Saline solution (0,85 % m/v)

A.3.1 Composition

Sodium chloride (NaCl)	(CAS RN® 7647-14-5)	8,5 g
Water		1 000 ml

A.3.2 Preparation

Dissolve the sodium chloride in the water.

Adjust the pH (6.6), if necessary, so that after sterilization, it is 7,0 ± 0,2 at 20 °C to 25 °C.

Dispense the solution into flasks or tubes of suitable capacity to obtain the portions necessary for the test.

Sterilize for 15 min in the autoclave (6.14) set at 121 °C.

Store the solution in closed flasks or tubes at 5 °C (6.4) for up to six months.

A.4 Performance testing for the quality assurance of the culture media

The definition of selectivity and productivity is specified in ISO 11133. In general, follow the procedures for performance testing described in ISO 11133, including the inoculum levels for the target and the non-target organisms. Table A.1 gives details of control strains to be used for performance testing of culture media specified in this document.

Table A.1 — Performance testing for the quality assurance of the culture media

Medium	Function	Incubation	Control strains	WDCM numbers[a]	Criteria[b]
Nutrient agar	Productivity	24 h ± 3 h/ 34 °C to 38 °C	*Salmonella* Typhimurium[c,d] *Salmonella* Enteritidis[c,d]	00031 00030	Good growth

[a] Refer to the reference strain catalogue at www.wfcc.info for information on culture collection strain numbers and contact details; WDCM: World Data Centre for Microorganisms.

[b] Growth is categorized as 0: no growth, 1: weak growth (partial inhibition), and 2: good growth (see ISO 11133).

[c] Some national restrictions and directions can require the use of a different serovar. Make reference to national requirements relating to the choice of *Salmonella* serovars.

[d] Strain free of choice; one of the strains shall be used as a minimum.

Annex B
(informative)

Probe-based multiplex real-time PCR assay for the identification of monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-)

B.1 General

This annex describes a probe-based multiplex real-time PCR method based on 5′- nuclease technology for the identification of the monophasic variant of *Salmonella* Typhimurium (1,4,[5],12:i:-) and the differentiation from other *Salmonella* non-Typhimurium monophasic serovars, by targeting the following genetic sequences:

— linkage between *fliA* and insertion sequence IS*200* (present in *Salmonella* Typhimurium (1,4,[5],12:i:1,2) and in monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-));

— linkage between *fljB* and *hin* (present in isolates expressing the second H phase antigen);

— linkage between *hin* and *iroB* (present in isolates expressing the second H phase antigen).

For the expression of the second H phase antigen, both amplification products, *fljB* – *hin* and *hin* – *iroB*, shall be detected. If one of the two products is not detected, expression of the second H phase antigen is interrupted.

The probe-based multiplex real-time PCR also contains a heterologous internal amplification control (IAC) based on the plasmid pUC18/19. By real-time PCR, a fragment spanning from M13pm18 to sequences of pBR322 is amplified. This sequence does not occur naturally.

NOTE Another IAC, with its specific primers and probe, can be used if it produces equivalent results.

B.2 Procedure

B.2.1 Principle

Three specific genetic sequences and a sequence of the IAC are amplified and detected by a probe-based multiplex real-time PCR method based on 5′- nuclease technology.

B.2.2 Reagents for PCR

B.2.2.1 General

See ISO 22174.

B.2.2.2 Primers and probes

Primers and probes are published in Reference [7]. The sequences are listed in Table B.1.

Table B.1 — Sequences of the primers and probes

Target sequence	Primer/probe name	Primer/probe sequence (5′-3′)	Amplicon size (bp)
fliA-IS*200*	fliA-IS200F	CAT TAC ACC TTC AGC GGT AT	254
	fliA-IS200R	CTG GTA AGA GAG CCT TAT AGG	
	fliA-IS200-probe2	FAM – CGG CAT GAT TAT CCG TTT CTA CAG AGG – BHQ1[a]	
fljB-hin	fljB-hinF	TGG TGC TGT TAG CAG AC	297
	fljB-hinR	TCA ACA CTA ACA GTC TGT CG	
	fljB-hin-probe	YY – AAC CGC CAG TTC ACG CAC – BHQ2[b]	
hin-iroB	hin-iroBF	GTG TGG CAT AAA TAA ACC GA	274
	hin-iroBR	AGG CTT ACC TGT GTC ATC CA	
	hin-iroB-probe	ROX – TAA CGC GCT CAC GAT AAG GC – BHQ2[c]	
IAC[d]	IAC-FW	GTC GGG AAA CCT GTC G	207
	IAC-RV	GCT CAC ATG TTC TTT CCT GC	
	IAC-probe	Cy5 – CGG GGA GAG GCG GTT– BHQ3[e]	

NOTE Other appropriate fluorophores and quenchers can be used.

[a] FAM: 6 Carboxyfluorescein, BHQ1: Black Hole Quencher 1™.[f]

[b] YY: Yakima Yellow™, BHQ2: Black Hole Quencher 2™.[f]

[c] ROX: Carboxy-X-rhodamine™, BHQ2: Black Hole Quencher 2™.[f]

[d] IAC: Internal amplification control.

[e] Cy5: Cyanine 5®, BHQ3: Black Hole Quencher 3™.[f]

[f] Products with trade names or trademarks are given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

B.2.3 Real-time PCR setup

B.2.3.1 Reaction setup

The total PCR volume is 25 µl per PCR reaction. The reagents for preparation of the reaction mix are listed in Table B.2.

Table B.2 — Preparation of the reaction mix

Reagent (stock concentration)	Final concentration	Volume per reaction (µl)[a]
10× PCR buffer containing no magnesium chloride	1×	2,5
dNTP-mix (each 2 mmol/l)	Each 200 µmol/l	2,5
Magnesium chloride (50 mmol/l) (CAS RN® 7786-30-3)	2,5 mmol/l	1,25
fliA-IS200F (20 pmol/µl)	0,4 pmol/µl	0,5
fliA-IS200R (20 pmol/µl)	0,4 pmol/µl	0,5
fljB-hinF (20 pmol/µl)	0,4 pmol/µl	0,5
fljB-hinR (20 pmol/µl)	0,4 pmol/µl	0,5
hin-iroBF (20 pmol/µl)	0,4 pmol/µl	0,5

NOTE For IAC, pUC 18 can also be used. 1 fg pUC 18/19 corresponds to approximately $3{,}4 \times 10^2$ copies.

[a] Volume per reaction depends on the concentration of the reagent in the stock solution and is determined by the final concentration.

[b] Performance characteristics of this assay were tested with the commercially available Platinum™ *Taq* Polymerase and 10× PCR buffer (Invitrogen). This information is given for the convenience of the user of this document and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to produce equivalent results.

Table B.2 *(continued)*

Reagent (stock concentration)	Final concentration	Volume per reaction (µl)[a]
hin-iroBR (20 pmol/µl)	0,4 pmol/µl	0,5
IAC-FW (20 pmol/µl)	0,4 pmol/µl	0,5
IAC-RV (20 pmol/µl)	0,4 pmol/µl	0,5
fliA-IS200-probe2 (5′FAM-3′BHQ1) (5 pmol/µl)	0,125 pmol/µl	0,625
fljB-hin-probe (5′YY-3′BHQ2) (10 pmol/µl)	0,25 pmol/µl	0,625
hin-iroB-probe (5′ROX-3′BHQ2) (10 pmol/µl)	0,25 pmol/µl	0,625
IAC-probe (5′Cy5–3′BHQ3) (10 pmol/µl)	0,25 pmol/µl	0,625
Taq Polymerase[b]	2 U	0,2
IAC-pUC19DNA	Approximately 10^5 copies	1,0
Cell suspension or DNA extract (9.1)		2,5
PCR grade water	Adjust to 25 µl	8,55

NOTE For IAC, pUC 18 can also be used. 1 fg pUC 18/19 corresponds to approximately 3,4 × 10^2 copies.

[a] Volume per reaction depends on the concentration of the reagent in the stock solution and is determined by the final concentration.

[b] Performance characteristics of this assay were tested with the commercially available Platinum™ *Taq* Polymerase and 10× PCR buffer (Invitrogen). This information is given for the convenience of the user of this document and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to produce equivalent results.

B.2.3.2 PCR controls

B.2.3.2.1 General

See ISO 22174.

B.2.3.2.2 Negative PCR control

Ultrapure water is used as negative control, see ISO 22174.

B.2.3.2.3 Positive PCR control

A cell suspension of *Salmonella* Typhimurium (1,4,[5],12:i:1,2) or a solution containing a defined sufficient amount and/or copy number of target DNA of *Salmonella* Typhimurium (1,4,[5],12:i:1,2) is used as a positive control (preparation see 9.1).

B.2.3.3 Temperature-time programme

Apply the temperature-time programme and number of cycles indicated in Table B.3 to 25 µl of the reaction mix, and the PCR controls.

Table B.3 — Temperature-time programme

Cycle step	Temperature[a] and time	No. of cycles
Activation/initial denaturation	95 °C for 2 min[b]	-
Denaturation Amplification	95 °C for 15 s 61 °C for 1 min	40 cycles

[a] The mentioned temperatures are the temperatures that should be effectively reached by the thermal cycler (6.12). These do not necessarily correspond with the programmed temperatures. If the thermal cycler shows a deviation after calibration, correct for this deviation.

[b] The activation time depends on the *Taq* Polymerase used.

B.3 Interpretation of PCR results

The PCR results shall be interpreted as follows:

a) positive reaction: fluorescence signal inclines exponentially during amplification and exceeds the threshold cycle crossing point.

b) negative reaction: the fluorescence signal does not exceed the threshold cycle crossing point.

The quantification cycle (C_q) value, or cycle threshold (C_t) value, is dependent on the thermal cycler model (6.12) and analysis software used for the assay and shall be determined for the users' own thermal cycler. A positive sample generates a typical amplification curve, with at least the exponential phase (see ISO 22174).

The positive (process) control (e.g. an isolate of biphasic *Salmonella* Typhimurium; see B.2.3.2.3) can help with the interpretation of the amplification curve and for setting the threshold to determine the C_t value of the target sequence of the isolate under analysis. Additionally, if an isolate is positive for different target sequences (see Table B.4), it is expected that the C_t value of the different targets, excluding IAC, is in the same order of magnitude. Further information and guidance for interpretation of real-time PCR results can be found in ISO 22174.

A sample is evaluated to be monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-) positive if the corresponding reaction *fliA*-IS*200* is positive and only one or none of the other two targets *fljB-hin* and *hin-iroB* are positive. The IAC can be positive or negative (see Table B.4).

A sample is evaluated to be *Salmonella* Typhimurium (1,4,[5],12:i:1,2) positive if the corresponding reactions *fliA*-IS*200* and additionally *fljB-hin* as well as *hin-iroB* are positive. The IAC can be positive or negative (see Table B.4).

A sample is evaluated to be *Salmonella* Typhimurium (1,4,[5],12:i:1,2) negative and monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-) negative if the corresponding reaction *fliA*-IS*200* is negative, irrespectively of the reaction results of *fljB-hin* and *hin-iroB*. The IAC shall be positive in cases where all targets are negative (see Table B.4). In cases where all targets, including the IAC, are negative, the reaction has failed and the run should be repeated for this sample.

Table B.4 — Interpretation of PCR results

Target sequence	1,4,[5],12:i:-	1,4,[5],12:i:-	1,4,[5],12:i:-	1,4,[5],12:i:1,2	Other serovars
fliA-IS*200*	+	+	+	+	-
fljB-hin	-	+	-	+	+ or -
hin-iroB	-	-	+	+	+ or -
IAC	+ or -	+ or -	+ or -	+ or -	+ or -[a]

[a] In cases where all targets are negative, the IAC shall be positive.

Annex C
(informative)

Agarose gel-based multiplex target PCR assay for the identification of monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-)

C.1 General

This annex describes an agarose gel-based multiplex PCR method for the identification of the monophasic variant of *Salmonella* Typhimurium (1,4,[5],12:i:-) and the differentiation from other *Salmonella* non-Typhimurium monophasic serovars, by targeting the following genetic sequences:

— The *fliB-fliA* intergenic region of the flagellin gene cluster, which contains the IS*200* element. A product of 1 000 bp is amplified in cases of *Salmonella* Typhimurium (1,4,[5],12:i:1,2) and monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-). For other *Salmonella* serovars, regardless of the first phase H-antigen, a 250 bp PCR product is amplified. The *fliB-fliA* intergenic region of these serovars does not have the IS*200* element.

— The *fljB* (present in isolates expressing second H phase antigen). The PCR product has a size of 1 389 bp.

In cases of *Salmonella* Typhimurium (1,4,[5],12:i:1,2), two PCR products with a size of 1 000 bp and 1 389 bp are amplified. For monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-), only one PCR product with a size of 1 000 bp is generated. For other serovars (including the ones sharing the H:i-antigen), PCR generates one amplicon of 250 bp. An amplicon of 1 389 bp can be generated.

NOTE Capillary electrophoresis instead of agarose gel electrophoresis can also be used to separate amplified products if it can be shown to produce equivalent results.

C.2 Procedure

C.2.1 Principle

Specific genetic sequences are amplified by multiplex PCR. Amplified products are separated by agarose gel electrophoresis and the size determined.

C.2.2 Reagents for PCR

C.2.2.1 General

See ISO 22174.

C.2.2.2 Primers

Primers are published in References [9] and [5]. The sequences are listed in Table C.1.

Table C.1 — Sequences of the primers

Target sequence	Primer name	Primer sequence (5′-3′)	Amplicon size (bp)
fliA-fliB	FFLIB	CTG GCG ACG ATC TGT CGA TG	1 000 or 250
	RFLIA	GCG GTA TAC AGT GAA TTC AC	
fljB	Primer Sense-59	CAA CAA CAA CCT GCA GCG TGT GCG	1 389
	Primer Antisense-83	GCC ATA TTT CAG CCT CTC GCC CG	

C.2.3 Multiplex PCR setup

C.2.3.1 Reaction setup

The total PCR volume is 25 µl per PCR reaction. The reagents for preparation of the reaction mix are listed in Table C.2.

Table C.2 — Preparation of the reaction mix

Reagent (stock concentration)	Final concentration	Volume per reaction (µl)[a]
2× Multiplex PCR Master Kit[b]	1×	12,5
FFLIB (10 pmol/µl)	0,4 pmol/µl	1
RFLIA (10 pmol/µl)	0,4 pmol/µl	1
Primer Sense-59 (10 pmol/µl)	0,2 pmol/µl	0,5
Primer Antisense-83 (10 pmol/µl)	0,2 pmol/µl	0,5
Cell suspension or DNA extract (see 9.1)		2
PCR grade water	Adjust to 25 µl	7,5

[a] Volume per reaction depends on the concentration of the reagent in the stock solution and is determined by the final concentration.

[b] Performance characteristics of this assay were tested with the commercially available Qiagen® Multiplex PCR Master Kit (Qiagen Hilden GmbH), which contains 6 mM Mg^{2+}. This information is given for the convenience of the user of this document and does not constitute an endorsement by ISO of this product. Equivalent products can be used if they can be shown to produce equivalent results.

C.2.3.2 PCR controls

C.2.3.2.1 General

See ISO 22174.

C.2.3.2.2 Negative PCR control

Ultrapure water is used as negative control, see ISO 22174.

C.2.3.2.3 Positive PCR control

A cell suspension of *Salmonella* Typhimurium (1,4,[5],12:i:1,2) or a solution containing a defined sufficient amount and/or copy number of target DNA of *Salmonella* Typhimurium (1,4,[5],12:i:1,2) is used for positive control (for preparation, see 9.1).

C.2.3.3 Temperature-time programme

Apply the temperature-time programme and number of cycles indicated in Table C.3 to 25 µl of the reaction mix, and the PCR controls.

Table C.3 — Temperature-time programme

Cycle step	Temperature[a] and time	No. of cycles
Activation/initial denaturation	95 °C for 15 min[b]	-
Denaturation	95 °C for 30 s	30 cycles
Annealing	64 °C for 30 s	
Amplification	72 °C for 1,5 min	

[a] The mentioned temperatures are the temperatures that should be effectively reached by the thermal cycler (6.12). These do not necessarily correspond with the programmed temperatures. If the thermal cycler shows a deviation after calibration, correct for this deviation.

[b] The activation time depends on the *Taq* Polymerase used.

C.2.4 Agarose gel electrophoresis

C.2.4.1 Reagents

The agarose gel electrophoresis can be carried out with tris-acetate-EDTA (TAE) buffer or tris-borate-EDTA (TBE) buffer. Solutions described in this annex usually do not need to be autoclaved.

C.2.4.1.1 Agarose, suitable for DNA electrophoresis and for the intended size separation of the DNA molecules.

C.2.4.1.2 Boric acid (H_3BO_3; CAS RN® 10043-35-3), for the TBE buffer system only.

C.2.4.1.3 Bromophenol blue sodium salt ($C_{19}H_9Br_4O_5SNa$; CAS RN® 34725-61-6) and/or **xylene cyanole FF** ($C_{25}H_{27}N_2O_6S_2Na$; CAS RN® 2650-17-1).

C.2.4.1.4 DNA molecular mass standard, e.g. a commercial preparation containing DNA fragments from very high to very low molecular mass.

C.2.4.1.5 Glacial acetic acid (CH_3COOH; CAS RN® 64-19-7), for the TAE buffer system only.

C.2.4.1.6 Ethylenediaminetetraacetic acid disodium salt (Na_2-EDTA) ($C_{10}H_{14}N_2O_8Na_2$; CAS RN® 6381-92-6).

C.2.4.1.7 Ethidium bromide (EtBr) ($C_{21}H_{20}N_3Br$; CAS RN® 1239-45-8) or other DNA gel stains.

Take care when using ethidium bromide solution as it is mutagenic/teratogenic. Other intercalating dyes can be used, by referring to the manufacturer's material safety data sheet.

C.2.4.1.8 Glycerol ($C_3H_8O_3$; CAS RN® 56-81-5).

C.2.4.1.9 Sodium acetate ($C_2H_3O_2Na$; CAS RN® 127-09-3), for the TAE buffer system only.

C.2.4.1.10 Hydrochloric acid, (HCl; CAS RN® 7647-01-0) = 37 % (volume fraction).

C.2.4.1.11 Sodium hydroxide (NaOH; CAS RN® 1310-73-2).

C.2.4.1.12 Tris(hydroxymethyl)-aminomethane (Tris) ($C_4H_{11}NO_3$; CAS RN® 77-86-1).

C.2.4.1.13 TAE buffer solution (1×), c(Tris) = 0,050 mol/l (C.2.4.1.12), $c(C_2H_3O_2Na)$ = 20 mmol/l (C.2.4.1.9), $c(Na_2\text{-}EDTA)$ = 0,001 mol/l (C.2.4.1.6). Adjust the pH to 8,0 with glacial acetic acid (C.2.4.1.5) or NaOH (C.2.4.1.11) at 20 °C to 25 °C. It is recommended to prepare the TAE buffer solution as a concentrated stock solution (maximum 50-fold concentrated). Discard it if a precipitate is visible. Dilution of the concentrated electrophoresis buffers can be carried out, immediately before its use, with non-sterile, (mono)-distilled or deionized water.

C.2.4.1.14 Tris/borate (TBE) buffer solution (0,5×), c(Tris) = 0,055 mol/l (C.2.4.1.12), c(boric acid) = 0,055 mol/l (C.2.4.1.2), $c(Na_2\text{-}EDTA)$ = 0,001 mol/l (C.2.4.1.6). Adjust the pH to 8,0 with HCl (C.2.4.1.10) or NaOH (C.2.4.1.11) at 20 °C to 25 °C. It is recommended to prepare the TBE buffer solution as a concentrated stock solution (maximum 10-fold concentrated). Discard it if a precipitate is visible. Dilution of the concentrated electrophoresis buffers can be carried out, immediately before its use, with non-sterile, (mono)-distilled or deionized water.

C.2.4.1.15 Sample loading buffer solution (5×), ϕ (glycerol) = 50 % (volume fraction; C.2.4.1.8), ρ (bromophenol blue) = 2,5 g/l (C.2.4.1.3) and/or ρ (xylene cyanol) = 2,5 g/l (C.2.4.1.3), dissolved in electrophoresis buffer solution.

NOTE Other concentrations of loading buffer solution or commercially prepared solutions can also be used. Some PCR buffers contain loading dye.

C.2.4.2 Equipment

The usual microbiological laboratory equipment (see ISO 7218) and, in particular, the following shall be used.

C.2.4.2.1 Microwave oven or **boiling water bath**, capable of operating at 95 °C to 100 °C.

C.2.4.2.2 Electrophoresis apparatus.

C.2.4.2.3 Ultraviolet (UV) transilluminator or **UV light box**.

C.2.4.2.4 Gel documentation system.

C.2.4.3 Safety and protection devices

WARNING — Ethidium bromide is a mutagen and toxic agent. Use it in compliance with the safety sheet provided and with protection devices (laboratory coats and gloves). UV light can cause damage to eyes. Therefore, use polymethylmethacrylate shields and protective glasses.

C.2.4.4 Agarose gel preparation and staining

The amplified PCR products should be detected using a 2,0 % (m/v) agarose gel. Weigh an appropriate amount of agarose (C.2.4.1.1) and add it to the electrophoresis buffer solution (C.2.4.1.13 or C.2.4.1.14). Allow the solution to boil in a microwave oven or in a water bath (C.2.4.2.1) until the agarose is completely dissolved, cool down the solution to approximately 60 °C and keep it at this temperature until usage. Prepare a gel support (gel tray) with a suitable sample comb placed in right position. Add ethidium bromide to a final concentration of 0,5 µg/ml (or equivalent DNA binding dye at the manufacturer's recommended concentration) before pouring the agarose solution onto the gel tray and allow the gel to solidify at room temperature (1 h is usually recommended).

Alternatively, the agarose gel can be stained after electrophoresis in a 0,5 µg/ml ethidium bromide aqueous solution (or equivalent DNA binding dye at the manufacturer's recommended concentration). For this, keep the gel in the dye at room temperature for 15 min to 50 min, preferably in the dark with gentle shaking.

If necessary, reduce the background staining by de-staining the gel in water for 10 min to 30 min.

NOTE To minimize the problems of ethidium bromide movement in the gel, some ethidium bromide can also be added to the electrophoresis (tank) buffer. After the gel electrophoresis, no de-staining step is usually required.

C.2.4.5 Agarose gel electrophoresis run

Following the amplification step add the loading buffer (C.2.4.1.15) to 10 µl reaction mix (see Table C.2) in the ratio 1:5 (e.g. add 2,5 µl of loading buffer to 10 µl of reaction mix) and mix.

NOTE Other concentrations of loading buffer solution can also be used. When the loading dye is already part of the PCR buffer, it does not need to be added separately.

Carefully remove the sample comb from the gel. Transfer the gel (with its gel tray) to the electrophoresis cell, so that the wells reside close to the cathode (negative electrode). Fill the cell with the electrophoresis buffer (C.2.4.1.13 or C.2.4.1.14). Overlay the gel with approximately 2 mm of the same buffer and load the samples using a micropipette. Apply the mixture to the sample slots (wells) with a micropipette. If the unknown samples are suspected to be too concentrated, also provide some dilutions of them to be loaded onto the gel.

To determine the size of the PCR products, add the loading buffer (C.2.4.1.15) and DNA molecular mass standard (C.2.4.1.4) in the proportion of 1:5. The DNA molecular mass standard is loaded on the gel at least before the first and after the last sample well.

Carry out the electrophoresis at room temperature at the appropriate voltage and power intensity (generally a maximum constant voltage of 5 V/cm, with respect to the distance between the electrodes, is recommended). Under the described conditions, DNA is negatively charged, so it migrates from the cathode to the anode. The electrophoresis time depends on the migration distance required, on the current generated by the power supply, the buffer used, the electro-endosmosis and the concentration of the agarose in the gel.

C.2.4.6 Agarose gel recording

Transfer the gel to the transilluminator surface (C.2.4.2.3), switch on the UV light and record the DNA fluorescence by photography or video-documentation.

C.3 Interpretation of PCR results

Samples showing amplification fragments of the expected size (see Table C.4) are considered as positive for related target sequences.

A sample is evaluated to be monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-) positive if the corresponding amplification product for *fliA-fliB* is 1 000 bp and for *fljB* no product was obtained (see Table C.4).

A sample is evaluated to be *Salmonella* Typhimurium (1,4,[5],12:i:1,2) positive if the corresponding amplification product for *fliA-fliB* is 1 000 bp and additionally for *fljB* is 1 389 bp (see Table C.4).

A sample is evaluated to be *Salmonella* Typhimurium (1,4,[5],12:i:1,2) negative and monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-) negative if the corresponding reaction *fliA-fliB* results in a 250 bp product, irrespectively of the reaction results of *fljB* (see Table C.4).

If there is any discrepancy from the result expected in the PCR controls, the run shall be repeated.

Table C.4 — Interpretation of PCR results (expected fragment sizes in bp)

Target sequence	1,4,[5],12:i:-	1,4,[5],12:i:1,2	Other serovars
fliA-fliB	1 000	1 000	250
fljB	—	1 389	1 389 or -
Key —: no PCR fragment			

Annex D
(informative)

Agarose gel-based single target PCR assay for the identification of monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-)

D.1 General

This annex describes an agarose gel-based single target PCR method for the identification of the monophasic variant of *Salmonella* Typhimurium (1,4,[5],12:i:-) and the differentiation from other *Salmonella* non-Typhimurium monophasic serovars, by targeting the following genetic sequences in three separated PCR reactions:

— linkage between *fliA* and insertion sequence IS*200* (present in *Salmonella* Typhimurium (1,4,[5],12:i:1,2) and in monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-); this will amplify a PCR product of 254 bp;

— linkage between *fljB* and *hin* (present in isolates expressing the second H phase antigen); this will amplify a product of 297 bp;

— linkage between *hin* and *iroB* (present in isolates expressing the second H phase antigen); this will amplify a product of 274 bp.

For the expression of the second H phase antigen, both amplification products, *fljB* – *hin* and *hin* – *iroB*, shall be detected. If one of the two products is not detected, expression of the second H phase antigen is interrupted.

In addition, each PCR reaction contains a heterologous internal amplification control (IAC) based on the plasmid pUC18/19. By PCR, a fragment spanning from M13pm18 to sequences of pBR322 is amplified. The PCR product has a size of 429 bp. This sequence does not occur naturally.

NOTE 1 Capillary electrophoresis instead of agarose gel electrophoresis can also be used to separate amplified products if it can be shown to produce equivalent results.

NOTE 2 Another IAC, with its specific primers, can be used if it produces equivalent results.

D.2 Procedure

D.2.1 Principle

Specific genetic sequences are amplified by single target PCR. Amplified products are separated by agarose gel electrophoresis and the size determined.

D.2.2 Reagents for PCR

D.2.2.1 General

See ISO 22174.

D.2.2.2 Primers

Primers are published in Reference [7]. The primers of the internal control are published in Reference [6]. The sequences are listed in Table D.1.

Table D.1 — Sequences of the primers

Target sequence	Primer name	Primer sequence	Amplicon size (bp)
fliA-IS*200*	fliA-IS200F	CAT TAC ACC TTC AGC GGT AT	254
	fliA-IS200R	CTG GTA AGA GAG CCT TAT AGG	
fljB-hin	fljB-hinF	TGG TGC TGT TAG CAG AC	297
	fljB-hinR	TCA ACA CTA ACA GTC TGT CG	
hin-iroB	hin-iroBF	GTG TGG CAT AAA TAA ACC GA	274
	hin-iroBR	AGG CTT ACC TGT GTC ATC CA	
IAC	HB10	ATT CCA CAC AAC ATA CGA GCC G	429
	HB11	GTT TCG CCA CCT CTG ACT TGA G	

D.2.3 Single target PCR setup

D.2.3.1 Reaction setup

The total PCR volume is 25 µl per PCR reaction. The reagents for preparation of the reaction mix for individual PCR reactions are listed in Tables D.2 to D.4.

Table D.2 — Preparation of the reaction mix for target sequence *fliA*-IS*200*

Reagent (stock concentration)	Final concentration	Volume per reaction (µl)[a]
10× PCR buffer containing no magnesium chloride	1×	2,5
dNTP-mix (each 2 mmol/l)	Each 200 µmol/l	2,5
Magnesium chloride (50 mmol/l) (CAS RN® 7786-30-3)	2,5 mmol/l	1,25
fliA-IS200F (20 pmol/µl)	0,4 pmol/µl	0,5
fliA-IS200R (20 pmol/µl)	0,4 pmol/µl	0,5
HB10 (20 pmol/µl)	0,4 pmol/µl	0,5
HB11 (20 pmol/µl)	0,4 pmol/µl	0,5
Taq Polymerase[b]	2 U	0,2
IAC - pUC19 DNA	Approximately 10^5 copies	1,0
Cell suspension or DNA extract (see 9.1)		2
PCR grade water	Adjust to 25 µl	13,55

NOTE For IAC, pUC 18 can also be used. 1 fg pUC 18/19 corresponds to approximately 3,4 × 10^2 copies.

[a] Volume per reaction depends on the concentration of the reagent in the stock solution and is determined by the final concentration.

[b] Performance characteristics of this assay were tested with the commercially available Platinum™ *Taq* Polymerase and 10× PCR buffer (Invitrogen). This information is given for the convenience of the user of this document and does not constitute an endorsement by ISO of this product. Equivalent products can be used if they can be shown to produce equivalent results.

Table D.3 — Preparation of the reaction mix for target sequence *fljB-hin*

Reagent (stock concentration)	Final concentration	Volume per reaction (µl)[a]
10× PCR buffer containing no magnesium chloride	1×	2,5
dNTP-mix (each 2 mmol/l)	Each 200 µmol/l	2,5
Magnesium chloride (50 mmol/l) (CAS RN® 7786-30-3)	2,5 mmol/l	1,25
fljB-hinF (20 pmol/µl)	0,4 pmol/µl	0,5
fljB-hinR (20 pmol/µl)	0,4 pmol/µl	0,5
HB10 (20 pmol/µl)	0,4 pmol/µl	0,5
HB11 (20 pmol/µl)	0,4 pmol/µl	0,5
Taq Polymerase[b]	2 U	0,2
IAC - pUC19 DNA	Approximately 10^5 copies	1,0
Cell suspension or DNA extract (see 9.1)		2
PCR grade water	Adjust to 25 µl	13,55

NOTE For IAC, pUC 18 can also be used. 1 fg pUC 18/19 corresponds to approximately 3,4 × 10^2 copies.

[a] Volume per reaction depends on the concentration of the reagent in the stock solution and is determined by the final concentration.

[b] Performance characteristics of this assay were tested with the commercially available Platinum™ *Taq* Polymerase and 10× PCR buffer (Invitrogen). This information is given for the convenience of the user of this document and does not constitute an endorsement by ISO of this product. Equivalent products can be used if they can be shown to produce equivalent results.

Table D.4 — Preparation of the reaction mix for target sequence *hin-iroB*

Reagent (stock concentration)	Final concentration	Volume per reaction (µl)[a]
10× PCR buffer containing no magnesium chloride	1×	2,5
dNTP-mix (each 2 mmol/l)	Each 200 µmol/l	2,5
Magnesium chloride (50 mmol/l) (CAS RN® 7786-30-3)	2,5 mmol/l	1,25
hin-iroBF (20 pmol/µl)	0,4 pmol/µl	0,5
hin-iroBR (20 pmol/µl)	0,4 pmol/µl	0,5
HB10 (20 pmol/µl)	0,4 pmol/µl	0,5
HB11 (20 pmol/µl)	0,4 pmol/µl	0,5
Taq Polymerase[b]	2 U	0,2
IAC - pUC19 DNA	Approximately 10^5 copies	1,0
Cell suspension or DNA extract (9.1)		2
PCR grade water	Adjust to 25 µl	13,55

NOTE For IAC, pUC 18 can also be used. 1 fg pUC 18/19 corresponds to approximately 3,4 × 10^2 copies.

[a] Volume per reaction depends on the concentration of the reagent in the stock solution and is determined by the final concentration.

[b] Performance characteristics of this assay were tested with the commercially available Platinum™ *Taq* Polymerase and 10× PCR buffer (Invitrogen). This information is given for the convenience of the user of this document and does not constitute an endorsement by ISO of this product. Equivalent products can be used if they can be shown to produce equivalent results.

D.2.3.2 PCR controls

D.2.3.2.1 General

See ISO 22174.

D.2.3.2.2 Negative PCR control

Ultrapure water is used as negative control, see ISO 22174.

D.2.3.2.3 Positive PCR control

A cell suspension of *Salmonella* Typhimurium (1,4,[5],12:i:1,2) or a solution containing a defined sufficient amount and/or copy number of target DNA of *Salmonella* Typhimurium (1,4,[5],12:i:1,2) is used for positive control (for preparation, see 9.1).

D.2.3.3 Temperature-time programme

Apply the temperature-time programme and number of cycles indicated in Table D.5 to 25 µl of the reaction mix, and the PCR controls.

Table D.5 — Temperature-time programme

Cycle step	Temperature[a] and time	No. of cycles
Activation/initial denaturation	95 °C for 5 min[b]	-
Denaturation	95 °C for 15 s	30 cycles
Annealing	61 °C for 1 min	
Amplification	72 °C for 1 min	

[a] The mentioned temperatures are the temperatures that should be effectively reached by the thermal cycler (6.12). These do not necessarily correspond with the programmed temperatures. If the thermal cycler shows a deviation after calibration, correct for this deviation.

[b] The activation time depends on the *Taq* Polymerase used.

D.2.4 Agarose gel electrophoresis

D.2.4.1 Reagents

The agarose gel electrophoresis can be carried out with tris-acetate-EDTA (TAE) buffer or tris-borate-EDTA (TBE) buffer. Solutions described in this annex usually do not need to be autoclaved.

D.2.4.1.1 Agarose, suitable for DNA electrophoresis and for the intended size separation of the DNA molecules.

D.2.4.1.2 Boric acid (H_3BO_3; CAS RN® 10043-35-3), for the TBE buffer system only.

D.2.4.1.3 Bromophenol blue sodium salt ($C_{19}H_9Br_4O_5SNa$; CAS RN® 34725-61-6) and/or **xylene cyanole FF** ($C_{25}H_{27}N_2O_6S_2Na$; CAS RN® 2650-17-1).

D.2.4.1.4 DNA molecular mass standard, e.g. a commercial preparation containing DNA fragments from very high to very low molecular mass.

D.2.4.1.5 Glacial acetic acid (CH_3COOH; CAS RN® 64-19-7), for the TAE buffer system only.

D.2.4.1.6 Ethylenediaminetetraacetic acid disodium salt (Na_2-EDTA) ($C_{10}H_{14}N_2O_8Na_2$; CAS RN® 6381-92-6).

D.2.4.1.7 Ethidium bromide (EtBr) ($C_{21}H_{20}N_3Br$; CAS RN® 1239-45-8) or other DNA gel stains.

Take care when using ethidium bromide solution as it is mutagenic/teratogenic. Other intercalating dyes can be used, by referring to the manufacturer's material safety data sheet.

D.2.4.1.8 Glycerol ($C_3H_8O_3$; CAS RN® 56-81-5).

D.2.4.1.9 Sodium acetate ($C_2H_3O_2Na$; CAS RN® 127-09-3), for the TAE buffer system only.

D.2.4.1.10 Hydrochloric acid, (HCl; CAS RN® 7647-01-0) = 37 % (volume fraction).

D.2.4.1.11 Sodium hydroxide (NaOH; CAS RN® 1310-73-2).

D.2.4.1.12 Tris(hydroxymethyl)-aminomethane (Tris) ($C_4H_{11}NO_3$; CAS RN® 77-86-1).

D.2.4.1.13 TAE buffer solution (1×), c(Tris) = 0,050 mol/l (D.2.4.1.12), c($C_2H_3O_2Na$) = 20 mmol/l (D.2.4.1.9), c(Na_2-EDTA) = 0,001 mol/l (D.2.4.1.6). Adjust the pH to 8,0 with glacial acetic acid (D.2.4.1.5) or NaOH (D.2.4.1.11) at 20 °C to 25 °C. It is recommended to prepare the TAE buffer solution as a concentrated stock solution (maximum 50-fold concentrated). Discard it if a precipitate is visible. Dilution of the concentrated electrophoresis buffers can be carried out, immediately before its use, with non-sterile, (mono)-distilled or deionized water.

D.2.4.1.14 Tris/borate (TBE) buffer solution (0,5×), c(Tris) = 0,055 mol/l (D.2.4.1.12), c(boric acid) = 0,055 mol/l (D.2.4.1.2), c (Na_2-EDTA) = 0,001 mol/l (D.2.4.1.6). Adjust the pH to 8,0 with HCl (D.2.4.1.10) or NaOH (D.2.4.1.11) at 20 °C to 25 °C. It is recommended to prepare the TBE buffer solution as a concentrated stock solution (maximum 10-fold concentrated). Discard it if a precipitate is visible. Dilution of the concentrated electrophoresis buffers can be carried out, immediately before its use, with non-sterile, (mono)-distilled or deionized water.

D.2.4.1.15 Sample loading buffer solution (5×), ϕ (glycerol) = 50 % (volume fraction; D.2.4.1.8)), ρ (bromophenol blue) = 2,5 g/l (D.2.4.1.3) and/or ρ (xylene cyanol) = 2,5 g/l (D.2.4.1.3), dissolved in electrophoresis buffer solution.

NOTE Other concentrations of loading buffer solution can also be used.

D.2.4.2 Equipment

The usual microbiological laboratory equipment (see ISO 7218) and, in particular, the following shall be used.

D.2.4.2.1 Microwave oven or **boiling water bath**, capable of operating at 95 °C to 100 °C.

D.2.4.2.2 Electrophoresis apparatus.

D.2.4.2.3 UV transilluminator or **UV light box.**

D.2.4.2.4 Gel documentation system.

D.2.4.3 Safety and protection devices

WARNING — Ethidium bromide is a mutagen and toxic agent. Use it in compliance with the safety sheet provided and with protection devices (laboratory coats and gloves). UV light can cause damage to eyes. Therefore, use polymethylmethacrylate shields and protective glasses.

D.2.4.4 Agarose gel preparation and staining

The amplified PCR products should be detected using a 2,0 % (m/v) agarose gel. Weigh an appropriate amount of agarose (D.2.4.1.1) and add it to the electrophoresis buffer solution (D.2.4.1.13 or D.2.4.1.14). Allow the solution to boil in a microwave oven or in a water bath (D.2.4.2.1) until the agarose is completely dissolved, cool down the solution to approximately 60 °C and keep it at this temperature until usage. Prepare a gel support (gel tray) with a suitable sample comb placed in right position.

Add ethidium bromide to a final concentration of 0,5 µg/ml (or equivalent DNA binding dye at the manufacturer's recommended concentration) before pouring the agarose solution onto the gel tray and allow the gel to solidify at room temperature (1 h is usually recommended).

Alternatively, the agarose gel can be stained after electrophoresis in a 0,5 µg/ml ethidium bromide aqueous solution (or equivalent DNA binding dye at the manufacturer's recommended concentration). For this, keep the gel in the dye at room temperature for 15 min to 50 min, preferably in the dark with gentle shaking.

If necessary, reduce the background staining by de-staining the gel in water for 10 min to 30 min.

NOTE To minimize the problems of ethidium bromide movement in the gel, some ethidium bromide can also be added to the electrophoresis (tank) buffer. After the gel electrophoresis, no de-staining step is usually required.

D.2.4.5 Agarose gel electrophoresis run

Following the amplification step add the loading buffer (D.2.4.1.15) to 10 µl reaction mix (Table D.2, D.3 or D.4) in the ratio 1:5 (e.g. add 2,5 µl of loading buffer to 10 µl of reaction mix) and mix.

NOTE Other concentrations of loading buffer solution can also be used. When the loading dye is already part of the PCR buffer, it does not need to be added separately.

Carefully remove the sample comb from the gel. Transfer the gel (with its gel tray) to the electrophoresis cell, so that the wells reside close to the cathode (negative electrode). Fill the cell with the electrophoresis buffer (D.2.4.1.13 or D.2.4.1.14). Overlay the gel with approximately 2 mm of the same buffer and load the samples using a micropipette. Apply the mixture to the sample slots (wells) with a micropipette. If the unknown samples are suspected to be too concentrated, also provide some dilutions of them to be loaded onto the gel.

To determine the size of the PCR products, add the loading buffer (D.2.4.1.15) and DNA molecular mass standard (D.2.4.1.4) in the proportion of 1:5. The DNA molecular mass standard is loaded on the gel at least before the first and after the last sample well.

Carry out the electrophoresis at room temperature at the appropriate voltage and power intensity (generally a maximum constant voltage of 5 V/cm with respect to the distance between the electrodes, is recommended). Under the described conditions, DNA is negatively charged, so it migrates from the cathode to the anode. The electrophoresis time depends on the migration distance required, on the current generated by the power supply, the buffer used, the electro-endosmosis and the concentration of the agarose in the gel.

D.2.4.6 Agarose gel recording

Transfer the gel to the transilluminator surface (D.2.4.2.3), switch on the UV light and record the DNA fluorescence by photography or video-documentation.

D.3 Interpretation of PCR results

Samples showing amplification fragments of the expected size (see Table D.6) are considered as positive for related target sequences. The IAC shall be positive (429 bp amplification product) in case of a negative target sequence amplification product.

A sample is evaluated to be monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-) positive if the corresponding amplification product for *fliA*-IS*200* is 254 bp and only one or none of the other two targets, *fljB-hin* (297 bp) and *hin-iroB* (274 bp), are positive (see Table D.6).

A sample is evaluated to be *Salmonella* Typhimurium (1,4,[5],12:i:1,2) positive if the corresponding amplification product for *fliA*-IS*200* is 254 bp and additionally *fljB-hin* (297 bp) and *hin-iroB* (274 bp) are positive (see Table D.6).

A sample is evaluated to be *Salmonella* Typhimurium (1,4,[5],12:i:1,2) negative and monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-) negative if the corresponding reaction *fliA*-IS*200* is negative, irrespective of the reaction results of *fljB-hin* or *hin-iroB* (see Table D.6).

If there is any discrepancy from the result expected in the controls (see Table D.6), the run shall be repeated.

Table D.6 — Interpretation of PCR results (expected fragment sizes in bp)

Target sequence	1,4,[5],12:i:-	1,4,[5],12:i:-	1,4,[5],12:i:-	1,4,[5],12:i:1,2	Other serovars
fliA-IS*200*	254	254	254	254	—
fljB-hin	—	297	—	297	297 or -
hin-iroB	—	—	274	274	274 or -
IAC	429 or -[a]	429 or -[a]	429 or -[a]	429 or -	429 or -[a]

Key

—: no PCR fragment

[a] In cases of a negative target, the IAC shall be positive.

Annex E
(informative)

Performance characteristics

E.1 Method(s) evaluation study

In 2018 and 2019, the three PCR assays described in Annexes B, C and D were tested in a method evaluation study.[8] For this, 172 different strains (target and non-target strains), isolated from different sources (food products, animals, animal feed, primary production samples and humans) were tested by two different laboratories (in Germany and in the Netherlands). For the inclusivity and the exclusivity testing of the method(s) evaluation study, the typing results of *Salmonella* found by slide agglutination were compared to the typing results found by each PCR method.

The following number of different strains were tested (*Salmonella* strains typed by slide agglutination):

— 53 (presumptive) monophasic *Salmonella* Typhimurium strains;

— 49 (biphasic) *Salmonella* Typhimurium strains;

— 43 strains of other *Salmonella* serovars (not monophasic *Salmonella* Typhimurium, not (biphasic) *Salmonella* Typhimurium);

— 27 strains of other *Enterobacteriaceae* (not *Salmonella* spp.).

The inclusivity and exclusivity results of the method evaluation study, determined by the two laboratories, are summarized in Tables E.1 and E.2.

It depends on the intended purpose for which the PCR assay is being applied, and its performance evaluated, whether only monophasic *Salmonella* Typhimurium is considered as target strain (and thus part of the inclusivity study) or if (biphasic) *Salmonella* Typhimurium is considered as target strain as well. For EC regulatory limits, it is important to know if the strain under analysis is the monophasic variant of *Salmonella* Typhimurium and not the monophasic variant of another *Salmonella* non-Typhimurium, because the regulatory limits are more stringent for *Salmonella* Typhimurium (including the monophasic variant) than for *Salmonella* non-Typhimurium strains. In this case, monophasic *Salmonella* Typhimurium as well as (biphasic) *Salmonella* Typhimurium can be considered as target strains and the three PCR assays described in Annexes B, C and D perform equally well for identification of monophasic *Salmonella* Typhimurium strains (see Table E.1). Only three strains serotyped as *Salmonella* Typhimurium with slide agglutination were typed as “other serovars” with the three PCR assays. These strains concern sequence type 36 (ST36), for which Reference [7] also reports deviating results for the real-time PCR assay.

If the aim is to identify whether the strain under analysis is either monophasic *Salmonella* Typhimurium or (biphasic) *Salmonella* Typhimurium, then *Salmonella* Typhimurium should be considered as non-target strain. For this purpose, the gel-based multiplex PCR (see Annex C) can be less specific for some strains than the other two PCR assays (see Table E.2). This can be explained by the fact that the gel-based multiplex PCR does not reflect all regions in the *flj* gene cluster, which are associated with second H phase flagellar antigen expression, while the multiplex real-time PCR as well as the gel-based single target PCR do target these regions. For this type of identification, it is recommended to use the multiplex real-time PCR (see Annex B) or the gel based single target PCR (see Annex D) for a higher specificity.

Table E.1 — Inclusivity and exclusivity results of the method evaluation study performed in two laboratories with the three PCR assays; considering both monophasic *Salmonella* Typhimurium (mSTm) and (biphasic) *Salmonella* Typhimurium (STm) as target strains and other *Salmonella* serovars and other *Enterobacteriaceae* as non-target strains

Method	Performance characteristic	Number of different strains	Total number of results	Inclusivity agreement	Inclusivity deviation	Exclusivity agreement	Exclusivity deviation
Multiplex real-time PCR (see Annex B)	Inclusivity (mSTm + STm)	102	204	198	6[a]	NA	NA
	Exclusivity	70	140	NA	NA	140	0
Gel-based multiplex PCR (see Annex C)	Inclusivity (mSTm + STm)	102	204	198	6[a]	NA	NA
	Exclusivity	70	140	NA	NA	140	0
Gel-based single target PCR (see Annex D)	Inclusivity (mSTm + STm)	102	204	198	6[a]	NA	NA
	Exclusivity	70	140	NA	NA	140	0

Key

NA: not applicable

[a] 3 different strains were identified as “other serovars” with the PCR assay by both laboratories, but as (biphasic) *Salmonella* Typhimurium with slide agglutination.